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SHORT COMMUNICATIONS

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Flow cell analysis of enzymatic RNA digests

HUNT[1] has reported that small labelled oligonucleotides can be detected in column eluents by mixing the eluent with a dioxane scintillator. This is an elaborate procedure which does not allow the recovery of the nucleotides. Therefore, a flow cell packed with anthracene was investigated. It can detect labelled oligonucleotides accurately in urea solution (pH 7.5) from enzymatic RNA digests and is therefore a simpler analytical tool. With this cell, the number of isomeric tri- and tetranucleotides in pancreatic ribonuclease digests of R17 and M12 RNA's were determined after a ribonuclease T_1 digestion, showing not reported similarities and differences between these bacteriophages.

All chemicals and pieces of equipment are commercially available and are described in detail by the manufacturers. Lucite flow cells from Packard Instrument Co. (La Grange, Ill.) were packed with crystalline anthracene (Packard Instrument Co.) whose fines had been removed by repeated decantations in 0.01 % Triton X-100. They were used for up to 6 months without repacking. When radioactive contamination of the cells occurred, most of it could be washed away with 10 mM NaOH, 0.01 % Triton X-100. For complete removal of the residual radioactivity, cells were washed with a detergent (Beckman Instruments Inc., part No.161 863, Palo Alto, Calif.). Connections, from the columns with ball and socket ends to the

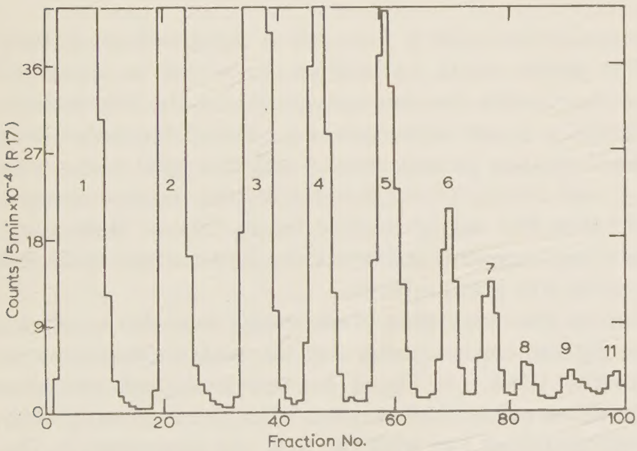

Fig. 1. Chromatography of the pancreatic ribonuclease digest of ^{32}P-labelled R17 RNA on a 0.5 cm × 20 cm DEAE-cellulose column. A linear gradient of 5 mM to 0.6 M NaCl (800 ml total volume), along with 7 M urea, 10 mM Tris (pH 7.5) and 0.01 % Triton X-100, was used. A flow rate of 22 ml/h was maintained with an accu-flow pump. A 2-ml flow cell at 6°, packed with anthracene, was used in a scintillation counter (3000 series from Packard Instrument Co.) equipped with a digital printing calculator. Discriminator and gain settings were optimized with the appropriate isotope present in the cells. Digital data were repetitively collected for a fixed-time interval and printed out in about 6 sec. After each print out, the scalers were reset and a new accumulation cycle began. ———, ^{32}P counts/5 min (preset time).

cell and to the pump, were with teflon fittings (22-AGW) and stainless tube swivel fittings all (except the cell) from Beckman Instruments Inc.

About 4 mg of ^{32}P-labelled R17 RNA ($1 \cdot 10^6$ disint./min) in 0.5 ml of 80 mM potassium phosphate (pH 7), isolated from R17 bacteriophage, were digested with pancreatic ribonuclease, then made 9 M in urea to increase density[2]. The digestion products were separated by chromatography. The elution pattern (Fig. 1), as recorded with a flow cell packed with anthracene, is similar to the pattern obtained by measurements of discrete collected samples. In Table I, the average number of mono- and oligonucleotide fragments per RNA molecule based on four experiments are not essentially different from reported data[3]. Therefore, at pH 7.5 in the presence of urea, flow cells packed with antracene do not sequester oligonucleotides and do not alter peak elutions significantly. With such flow cells different RNA's are comparable through the chromatographic analysis of their pancreatic digests if each RNA is labelled with a different isotope. Thus, [^3H]adenine M12 RNA was compared with R17 [^{32}P]RNA (not shown).

TABLE I

COLUMN ANALYSIS AT pH 7.5 OF R17 RNA DIGESTED WITH PANCREATIC RIBONUCLEASE

Peak No.:	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*11*
R17 fragments as determined with the cell	917	420	210	93	56	21	13	3.2	4.2	2.0
R17 fragments from reported data[3]	894	398	183	91	52	21	13	3	4	2

The detection of oligonucleotides with a flow cell is independent of their base composition. Similarly to purine tracts, pyrimidine tracts can be separated and monitored at pH 7.5, as shown with the chromatography of the ribonuclease T_1 digest of 4 mg of R17 [^{32}P]RNA ($2 \cdot 10^6$ disint./min) on a DEAE-Sephadex A-25 column. Elution was with NaCl varying linearly from 5 mM to 0.55 M along with 7 M urea, 10 mM Tris (pH 7.5) and 0.01 % Triton X-100. Eighteen peaks were separated. The last three represent long R17 oligonucleotide tracts. Two of them must be the 17 and the 21 nucleotide long fragments coding for the first 6 amino acids and the 89th to 95th amino acid of the R17 coat protein[4].

The flow cell is adequate for the monitoring of nucleotide solutions at pH 7.5 but not at pH 3.3, as shown by the elution pattern of the rechromatography at pH 3.3 of the trinucleotide fraction (Peak 3 of Fig. 1). Between each peak and after the last one the background level was not reached as it was when the chromatography was performed under identical conditions but with the flow cell disconnected. The number of trinucleotides as determined by the radioactivity detected with the cell was found to be different from the number determined with the cell disconnected (*cf.* ref. 3).

This indicates that at pH 3.3, nucleotides interact with the flow cell or its contents. This effect is more pronounced with nucleotides of longer chain length. When Peak 7 of Fig. 1 is rechromatographed at pH 3.3, the elution pattern (Fig. 2)

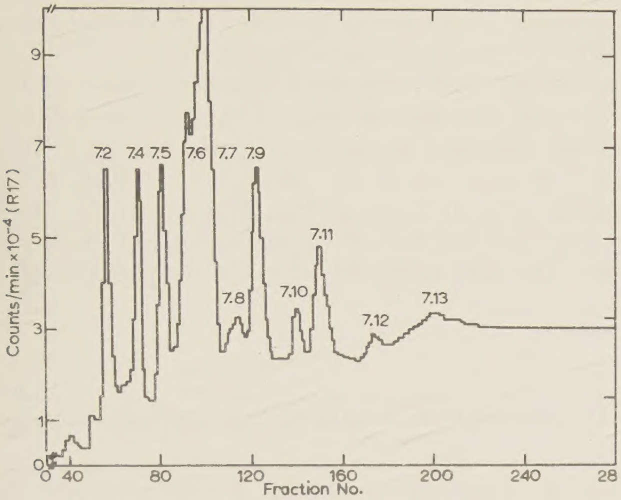

Fig. 2. Rechromatography of the heptanucleotide fraction of a pancreatic ribonuclease digest of R17 RNA on a 0.5 cm × 30 cm DEAE-cellulose column. A linear gradient of 10 mM to 0.3 M NaCl (600 ml total volume), along with 7 M urea, 0.1 M formic acid (pH 3.3) and 0.01 % Triton X-100, was used. A flow rate of 23 ml/h was maintained. A 1-ml flow cell at 6° was used. ———, ^{32}P counts/5 min (preset time).

shows that although peaks are resolved from one another, the background is very high. After its subtraction, the number of nucleotides in each peak can be roughly estimated. No appreciable contamination of each peak by the nucleotides retained in the flow cell seems to occur as determined by their base composition.

In the study of R17 and M12 RNA's[3], the enumeration of the isomeric tri- and tetranucleotides was not determined. This is now reported using the cell to monitor the radioactive pH 7.5 column effluent. R17 and M12 [^{32}P]RNA were digested with pancreatic ribonuclease and the products separated by chromatography on DEAE-cellulose columns[3]. Of these, the isomeric mixtures of the tri- and tetranucleotides were desalted, digested with ribonuclease T_1 and chromatographed on DEAE-cellulose in the presence of 7 M urea, 0.01 % Triton X-100, 10 mM Tris (pH 7.4) with a linear sodium acetate gradient from 10 mM to 0.3 M. About 50 μg of R17 ^{32}P-labelled (A,G)Cp were digested with ribonuclease T_1 and chromatographed. The five peaks of the elution patterns were G > p, Cp, A–G > p and Gp, A–Cp and A–Gp, respectively. The percentage of G–A–Cp in (G,A)Cp was 50.9 %. In other experiments, the elution patterns were as above, except that a small sixth peak made of (A,G)Cp was present in the amount of 2 % of the total radioactivity. About 50.8 % for R17 G–A–Cp and 49.2 % for R17 A–G–Cp were the averages for three experiments. Similarly, M12 (A,G)Cp, M12 (A,G)Up and R17 (A,G)Up were analysed. There was 42.5 % and 57.5 % of M12 G–A–Cp and A–G–Cp, 63.7 % and 36.3 % of M12 G–A–Up and A–G–Up, 55.7 % and 44.3 % of G–A–Up and A–G–Up, respectively, as determined by averaging three experiments.

M12 (A,G,G)Up was digested and chromatographed as above. The five peaks of the elution pattern were G> p, Up, A–G> p and G–G> p and Gp, A–Up, (G,A)-G > p and A–Gp and G–Gp, respectively. Similarly M12 (A,G,G)Cp, R17 (A,G,G)

Up and R17 (A,G,G)Cp were analysed. There was 37 % of M12 G–G–A–Up against 37.4 % in R17 and 21.5 % of M12 G–G–A–Cp against 21.2 % in R17.

R17 (A,A,G)Up was digested and chromatographed. The seven peaks of the elution pattern were G > p, Up, A–G > p and Gp, A–Up, A–A–G > p and A–Gp, A–A–Up and A–A–Gp, respectively. Similarly M12 (A,A,G)Up and R17 (A,A,G)Cp were analysed. There was 24.5 % of R17 A–A–G–Up, 34.5 % of R17 A–G–A–Up and 41 % of R17 G–A–A–Up against 21 %, 34 % and 45 % in M12 respectively. There was 30.1 % of R17 A–A–G–Cp, 30.7 % of R17 A–G–A–Cp and 39.2 % of G–A–A–Cp. M12 (A,A,G)Cp was not digested. The same results for R17 (A,A,G)Cp and for M12 (A,A,G)Cp were assumed.

TABLE II

THE NUMBER OF TRI- AND TETRANUCLEOTIDES ISOMERS IN R17 AND M12 PANCREATIC RIBONUCLEASE RNA DIGESTS

	G–A–Cp	*A–G–Cp*	*A–A–G–Cp*	*A–G–A–Cp*	*G–A–A–Cp*	*G–G–A–Cp*	*(G,A)G–Up*
M12	22	29	5	5	7	3	12
R17	27	26	5	5	6	3	11
	G–A–Up	*A–G–Up*	*A–A–G–Up*	*A–G–A–Up*	*G–A–A–Up*	*G–G–A–Up*	*(G,A)G–Up*
M12	33	19	4	6	9	6	10
R17	26	20	5	7	9	6	10

If 51 (A,G)Cp, 52 (A,G)Up, 17 (A,A,G)Cp, 15 (A,G,G)Cp, 18 (A,A,G)Up and 15 (A,G,G,)Up are assumed to have been in M12 against 53, 46, 16, 14, 21 and 16 in R17 (*cf*. ref. 3) the number of positional isomers was as in Table II; 26 R17 G–A–Up against 33 in M12 and 27 R17 G–A–Cp against 22 in M12 were significant differences between R17 and M12 RNA's.

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